The Effects of Acute Social Stress on Epidermal Langerhans' Cell Frequency and Expression of Cutaneous Neuropeptides

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Psychological stress is believed to exacerbate inflammatory skin disease but the underlying mechanisms are poorly understood. We investigated the impact of acute social stress –Trier public speaking test – on: epidermal Langerhans' cell (LC) frequency; and cutaneous nerve fiber expression of protein gene product (PGP) 9.5 and calcitonin gene-related peptide (CGRP). Thirty-six healthy volunteers each had a pair of baseline 6 mm biopsies taken from sun-protected buttock skin. A second pair of biopsies was taken from contralateral buttock 4 hours ($n=5$) or 24 hours ($n=15$) after the Trier stressor. Controls ($n=16$) did not perform the Trier and had biopsies 24 hours apart. One of each pair of biopsies (baseline; 4 or 24 hours) was processed for counts of epidermal CD1a$^+$ LC; the other examined for PGP 9.5 and CGRP expression. We observed a significant ($P<0.01$) 16.4% reduction in epidermal LC frequency 24 hours post-stressor as compared with baseline; there was no significant change from baseline in non-stressed controls. At 24 hours, PGP 9.5 and CGRP were increased ($P=0.025$) and reduced ($P=0.03$), respectively, from baseline in the stressed group compared with controls. These data suggest that acute social stress reduces epidermal LC frequency and modulates cutaneous neuropeptide expression thereby supporting the concept of a "brain–skin" axis.

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INTRODUCTION

Psychological stress may trigger a physiological ''fight or flight'' or stress response (Dhabhar and McEwen, 1997). Acute stress, defined as lasting for minutes to hours, is a conserved evolutionary mechanism known to stimulate and enhance immune responses (Dhabhar, 2002). Such responses may be deleterious for inflammatory skin diseases such as psoriasis, eczema, and acne, but beneficial for survival in terms of wound healing or resistance to infection (Dhabhar, 2002). The mechanisms underlying acute stress-induced exacerbation of inflammatory skin disease are not well understood, although it is likely that the ''brain–skin'' axis, described as the interactions between psyche, immune system, and cutaneous inflammation, plays a key role.

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Abbreviations: CGRP, calcitonin gene-related peptide; DC, dendritic cells; LC, Langerhans' cell; PGP, protein gene product; PBS, phosphate-buffered saline; PSWQ, Penn State Worry Questionnaire; VAS, visual analogue scale

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Langerhans' cells (LCs), members of a wider family of dendritic cells (DCs), are a unique population of leukocytes that reside in the epidermis and regulate cutaneous immune responses (Cumberbatch *et al.*, 2000). Their responsibilities include sampling of the external environment for challenges and delivery of information (antigen) to T cells within skin-draining lymph nodes. The exact role of LC is currently the source of speculation, the traditional view being that they contribute to initiation of adaptive cutaneous inflammation. Recent work has shown that the afferent phase of contact hypersensitivity is enhanced in Langerin–diphtheria toxin subunit A-transgenic mice that lack epidermal LC (Kaplan *et al.*, 2005). This observation implies that LC may play a regulatory or suppressive role in cutaneous immunity (Kaplan *et al.*, 2005; Ritter and Osterloh, 2007).

It is known that LC may be closely associated anatomically with epidermal nerve fibers and in contact with axons via their cell body (Hosoi *et al.*, 1993; Gaudillere *et al.*, 1996) and dendrites (Gaudillere *et al.*, 1996). Studies in mice have shown that acute stress causes reduction in epidermal LC frequency (Hosoi *et al.*, 1998; Ruiz *et al.*, 2003) and modulation of LC morphology (Kawaguchi *et al.*, 1997; Hosoi *et al.*, 1998; Ruiz *et al.*, 2003).

Calcitonin gene-related peptide (CGRP), a 37-amino-acid sensory neuropeptide, is distributed throughout the peripheral and central nervous systems (Rosenfeld *et al.*, 1983).

Located in unmyelinated C and myelinated A delta sensory nerve fibers in the periphery, CGRP, and substance P are co-stored in single secretory granules (Gibson *et al.*, 1984). CGRP receptors have been identified on many different cells, including LC (Asahina *et al.*, 1995a, b), suggesting that CGRP may have immunomodulatory potential. Exposure of LC to CGRP *in vitro* leads to inhibition of antigen presentation (Hosoi *et al.*, 1993; Asahina *et al.*, 1995b) possibly by limiting production of cytokines such as IL-1β and -12; augmenting production of IL-10 (Hosoi *et al.*, 1995; reviewed in Downing and Miyan, 2000); and inhibiting upregulation of costimulatory molecule B7-2 (CD86; Torii *et al.*, 1997). Topical application of CGRP to the abdominal skin of mice causes a significant reduction in epidermal LC frequency (Gillardon *et al.*, 1995; reviewed in Seiffert and Granstein, 2002). This observation suggests that CGRP may play a role in modulation of epidermal LC frequency.

Protein gene product (PGP) 9.5, a constitutive cytoplasmic neuron-specific protein, is present in large amounts in neurons of human central and peripheral nervous systems (Thompson *et al.*, 1983). The availability of anti-PGP 9.5 antibody provides for a well-characterized method to identify reliably most if not all nerve fibers in human skin (Kelly *et al.*, 2005).

In vitro and animal studies have investigated the effect of stress on epidermal LC (Kawaguchi *et al.*, 1997; Hosoi *et al.*, 1998; Ruiz *et al.*, 2003) and the expression of cutaneous neuropeptides (Kawaguchi *et al.*, 1997; Ruiz *et al.*, 2003). Studies measuring the effect of psychological stress on human skin have focused either on barrier function and/or circulating levels of cytokines (Altemus *et al.*, 2001; Garg *et al.*, 2001; Muizzuddin *et al.*, 2003). Stress induced in medical students by university examinations (Garg *et al.*, 2001) and psychological stress from marital separation delays cutaneous barrier recovery after tape-stripping (Muizzuddin *et al.*, 2003). Altemus *et al.* (2001) observed that acute psychosocial and sleep deprivation stress disrupts skin barrier function in women and also increases circulating levels of IL-1β, IL-10, and tumor necrosis factor-α. This is consistent with other studies in which secretion of tumor necrosis factor-α and IL-10, and to a lesser degree IL-1β, into the bloodstream during and following exercise stress have been reported (Weinstock *et al.*, 1997; Ostrowski *et al.*, 1999).

In this study, we examined the effect of acute experimental social stress (in the form of the Trier paradigm) on: epidermal LC frequency; and expression of neuropeptides in human skin.

RESULTS

Participant characteristics

Thirty-six healthy volunteers (19 men and 17 women; mean age, 24; range, 18–45 years) participated in the study between August 2005 and July 2006. All healthy volunteers had a pair of 6 mm punch biopsies taken from sun-protected buttock skin at baseline. A second pair of biopsies was taken from contralateral buttock skin 4 hours (five men; mean age, 24.8; range, 22–27) or 24 hours (seven men and eight women; mean age, 23.9; range, 18–45) after exposure to the Trier social stressor. Control (non-stressed) volunteers (seven men and nine women; mean age, 23.3; range 18–40) were not exposed to the Trier test and had biopsies taken at baseline and at 24 hours.

All subjects were naive in that they neither had previous volunteer experience of skin biopsies nor the Trier test. Apart from women ($n=9$) taking the oral contraceptive pill, volunteers did not take regular medication.

Psychological data

Using the documented cutoffs on the worry measure to differentiate between low, nonpathological worry (Penn State Worry Questionnaire (PSWQ) <60) and high, pathological worry (PSWQ >60; Meyer *et al.*, 1990), all participants scored in the low, nonpathological worry category. These data are consistent with scores expected from healthy volunteers (Meyer *et al.*, 1990; Richards *et al.*, 2005). There was no difference in level of worry between the stressed and control groups.

Post-stressor assessments

Blood pressure and pulse measurements. A small, nonsignificant increase of mean systolic ($P=0.2$) and diastolic ($P=0.4$) blood pressures from baseline was observed post-stressor in the stressed group (24 hours Trier; $n=9$), whereas a small but nonsignificant reduction of these two measures (systolic: $P=0.2$; diastolic: $P=0.4$) was noted in the control (non-stressed) group ($n=16$). There was no significant change in mean pulse rate from baseline in either the 24 hours stressed group ($P=0.07$) or the control group ($P=0.3$). Complete data were not recorded for the 4 hours stress group and in six subjects in the 24 hours stress group (24 hours Trier).

Visual analogue scale. At the end of the first study visit, following the stressor (24 hours Trier) or baseline biopsies (controls), participants used a linear visual analogue scale (VAS) to record the amount of stress experienced by participation in the study (''0'' – ''not at all'' to 100 – ''very much''). The mean VAS scores for subjective stress for the 24 hours stress group ($n=12$) and controls ($n=16$) were significantly different (58.4 ± 4.3 and 31 ± 6.3, respectively; $P=0.002$) indicating that participation in the Trier was perceived as stressful.

LC migration induced by acute psychosocial stress. Changes in the frequency of $CD1a^+$ cells were examined in epidermal sheets derived from biopsies obtained before (0 hour) and either 4 or 24 hours following exposure to the stressor, or 24 hours later with no intervening stressor (control group). Baseline (0 hour) epidermal LC frequencies in biopsies taken before volunteers ($n=20$) being subjected to the stressor, or at 0 hour for the control, non-stressed group ($n=15$), ranged from 624 to 1,217 LC per mm^2 (Figure 1); a range similar to that reported previously for resting frequency of epidermal LC in volunteers of this age group (Cumberbatch *et al.*, 1999). No significant differences in epidermal LC frequencies were observed 4 hours ($n=5$) after exposure to the stressor

compared with baseline (0 hour) values. However, 24 hours after exposure to the stressor, most individuals showed a reduction in epidermal LC frequency, with 13 out of 15 volunteers showing a decline in LC frequency of 10% or more. Overall a significant (16.4±2.6%, (mean±SE); $P<0.001$) reduction in epidermal LC numbers was observed for this group when compared with baseline values. This effect was not observed in the control group that had biopsies taken at the same time points but without exposure to the Trier stressor. For this group, only 2 out of 15 volunteers showed a reduction in epidermal LC frequency of ⩾10%. In fact, 7 out of 15 showed an increase in epidermal LC number of ⩾10% or more. Overall there was a small increase in epidermal LC frequency (mean 6.3±4.88%, (mean±SE)) but this was statistically insignificant.

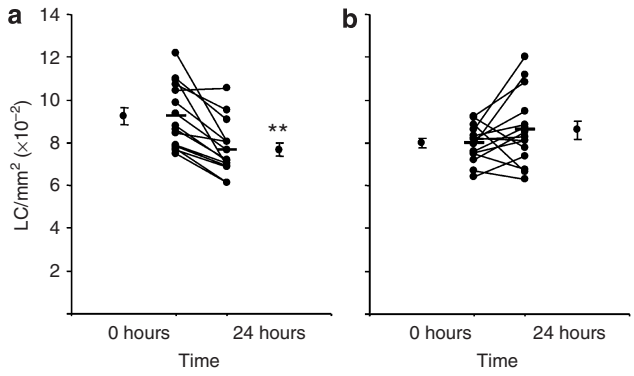

Figure 1. Acute psychosocial stress reduces epidermal CD1a$^+$ LC frequency. Two cohorts of normal volunteers were recruited to the study (**a** and **b**). In both cohorts, a baseline (0 hour) biopsy was obtained from sun-protected buttock skin. (**a**) Cohort: volunteers were then exposed to the Trier test, as a form of acute psychosocial stress, and a second biopsy was taken from contralateral buttock skin 24 hours later ($n=15$). (**b**) Cohort: volunteers had a second biopsy from contralateral buttock 24 hours after baseline but were not exposed to the Trier psychosocial stressor ($n=15$). This was the control group. LC frequencies were assessed by indirect immunofluorescence staining of epidermal sheets for CD1a expression. Results are shown as the frequencies of LC per mm^2 per individual derived from examination of 50 high-power fields per sample. (**a**) Acute psychosocial stress produced a significant reduction in epidermal LC frequency at 24 hours compared with baseline (**$P<0.001$; randomized analysis of variance). (**b**) There was no significant change of LC frequency at 24 hours compared with baseline in the control group.

The change in epidermal LC frequency in the 24 hours stress group was compared with the controls. It is important to note that although the baseline values for this study are within the range seen previously for healthy volunteers in this age group (Cumberbatch *et al.*, 1999), the individual group means are significantly different; the randomization of the participant selection may account for this effect. The analysis performed on the data takes this into account and calculates what the means would be if the baseline for both groups was the same. This reveals a very significant ($P<0.001$) change in LC frequency between groups at 24 hours.

Stress increases expression of PGP 9.5. We observed PGP 9.5-immunoreactive nerve fibers in both the epidermis and dermis. In the 4 hours stress group ($n=5$), the trend of increased percentage area of PGP 9.5+ nerve fibers at 4 hours after exposure to stressor as compared with baseline did not achieve statistical significance ($P=0.16$; 95% confidence interval: −0.2 to 0.05). In the 24 hours stress group, PGP 9.5+ nerve fibers were increased on average by 0.163 percentage area (95% confidence interval: 0.02–0.30) more than in the control group and there was a significant ($P=0.025$) difference between the two groups in terms of increase in PGP 9.5+ nerve fibers from baseline (Table 1).

Stress reduces the expression of CGRP. CGRP+ nerve fibers were observed in the epidermis, at the dermo-epidermal junction and in a perivascular distribution in the papillary dermis. In keeping with studies performed in human skin (Hosoi *et al.*, 1993), we observed a close association between CGRP+ nerve fibers and epidermal LCs in both stressed and control volunteers (Figure 2).

In contrast to PGP 9.5 expression, we observed a nonsignificant ($P=0.38$; 95% confidence interval: −0.03 to 0.06) trend of reduced expression of CGRP 4 hours post-stressor ($n=5$). At 24 hours, CGRP+ nerve fibers in the stressed group decreased on average by 0.033 percentage area (95% confidence interval: 0.0003–0.07) more than in the control group and there was a significant ($P=0.03$) difference between the two groups in terms of reduction of CGRP+ nerve fibers from baseline (Table 1). We interpret these findings as indicative of release of CGRP from nerve endings.

Table 1. Mean change of PGP 9.5+ and CGRP+ nerve fibers between 24 h stress group (n=15) and non-stressed controls (n=16)

	Non-stressed controls[1]	24 h stress group[1]	Difference[2] (95% CI)	*P*-value[3]
Mean change of PGP 9.5 at 24 h	−0.023 (0.03)	+0.140 (0.06)	0.163 (0.02–0.30)	0.025
Mean change of CGRP at 24 h	0.0003 (0.005)	0.033 (0.01)	0.033 (0.0003–0.07)	0.03

CI, confidence interval; CGRP, calcitonin gene-related peptide; PGP 9.5, protein gene product 9.5.
Units: PGP 9.5 and CGRP+ nerve fibers: % area.
[1]Mean±SEM.
[2]Difference between 24 h stress group and non-stressed controls in terms of change in mean percentage area of PGP 9.5+ or CGRP+ nerve fibers from baseline.
[3]*P*-values were obtained using an independent two-sided *t*-test (equal variances not assumed).

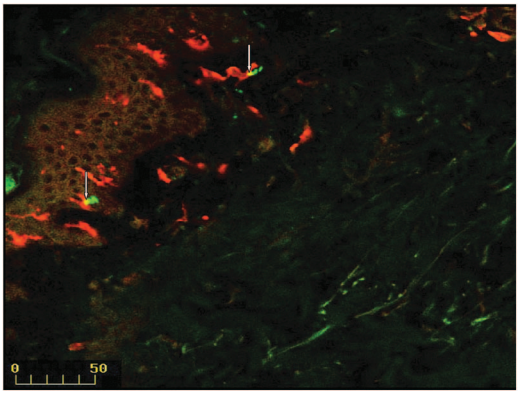

Figure 2. Co-localization of epidermal LCs and CGRP+ nerve fibers in normal human skin. Confocal image of epidermal LCs (red) and CGRP+ nerve fibers (green) shows intimate relationship between LCs and CGRP expressing nerve fibers (Bar = 0–50 μm).

DISCUSSION

These investigations provide early evidence that acute experimental social stress is associated with a reduction in frequency of epidermal LC, accompanied by altered expression of cutaneous neuropeptides. In the absence of an appropriate precedent, the time points chosen to assess the impact of the stressor on epidermal LC frequency and PGP 9.5 and CGRP immunoreactivity were informed by studies in humans of the tempo of LC migration in response to cytokine stimuli (reviewed in Griffiths *et al.*, 2005), and previously reported studies in mice (Kawaguchi *et al.*, 1997; Hosoi *et al.*, 1998; Ruiz *et al.*, 2003). In the latter investigations, stress was induced by immobilization with a duration of 2–8 hours or overcrowded housing of mice. Factors to be considered when translating these observations and data to healthy volunteers include: (i) the brief duration of the modified Trier paradigm as compared with the duration of stressor used in murine studies; (ii) reasonable period of time to detain participants in the research department; and (iii) psychological characteristics and stress perception in human subjects. On the basis of those considerations, we concluded that assessment of changes at 24 hours after exposure to stressor might be most relevant and most informative. We also investigated a small cohort of subjects from whom biopsies were taken at an earlier time point (4 hours post-stressor).

We did not observe a difference between the groups in terms of pathological worry. This assessment was important because high worriers may be more likely to be stressed/distressed by the study protocol.

The VAS scores of subjective stress experienced by participants, as well as blood pressure and pulse rate measurements suggest, as expected, that the group exposed to the Trier stressor experienced more stress than the control group. The small, nonsignificant change in vital signs was in contrast to our previous work that showed a significant increase in pulse rate and blood pressure in patients with psoriasis and in controls following exposure to the Trier stressor (Richards *et al.*, 2005). Similar to our previous study, we measured blood pressure and pulse rate pretesting and immediately following the stressor or baseline biopsies. Ideally, continuous electrocardiogram monitoring and/or measurement of circulating levels of catecholamines and cortisol should be performed to assess the systemic effect of the stressor.

The important finding was that 24 hours after Trier stress, there was a significant reduction in epidermal LC frequency. These data are consistent with studies in mice in which it was found that stress caused by overcrowding, restraint, or immobilization was associated with a reduced density of LC (Hosoi *et al.*, 1998; Ruiz *et al.*, 2003), and altered morphology (decreased number of dendrites and cell size; Kawaguchi *et al.*, 1997; Hosoi *et al.*, 1998). The conclusion drawn is that acute social stress has effects on human epidermal LC numbers comparable with those observed previously in mice. We propose that this reduction in LC numbers is a consequence of mobilization and the migration of LC from epidermis to regional lymph nodes. Although it was not possible to measure directly the accumulation of cells within lymph nodes, such an interpretation is consistent with what is known of LC migration provoked by other stimuli in both mouse and man (reviewed in Griffiths *et al.*, 2005).

Neuropeptides, such as CGRP, present in sensory (afferent) neurons are synthesized in the cell bodies of the spinal ganglia and transported along the axon to the periphery for release (reviewed in Shepherd *et al.*, 2005). It is known that sensory (afferent) neurons may also have efferent function (reviewed in Foreman, 1987a, b). Consequently, the significant reduction in CGRP immunoreactivity observed 24 hours post-stressor is consistent with the direct effect of stress-induced neuronal activation and subsequent CGRP release from secretory granules in the sensory (afferent) neurons. We suggest that the earlier time point, 4 hours post-stressor, may be too early to detect significant changes in CGRP+ nerve fiber density, although the small sample size ($n = 5$) may also have played a part. Ruiz *et al.* (2003) examined the effect of immobilization stress on murine epidermal LC, and neuropeptides, including CGRP. They did not, however, report data from the control group (non-stressed, non-infected mice) and consequently, these data did not inform the interpretation of our results.

In contrast, Kawaguchi *et al.* (1997) reported a significant increase in intensity of CGRP+ nerve fibers (visualized by immunofluorescence) in mice stressed by immobilization for 8 hours as compared with non-stressed controls. Furthermore, although there were no differences between stressed and non-stressed mice in terms of epidermal LC frequency, morphological changes in LC were observed in the skin of stressed mice (Kawaguchi *et al.*, 1997). It is of interest that in those studies increased CGRP expression occurred in the absence of altered LC frequency; data that are therefore inconsistent with the findings of Hosoi *et al.* (1998), and with the results reported here.

The mechanisms resulting in stress-induced reduction of epidermal LC frequency are currently not known. It is possible that CGRP itself may, at least in part, be responsible for changes in LC frequency given that topical application of

CGRP in mice has been shown to reduce the numbers of epidermal LC (Gillardon *et al.*, 1995; reviewed in Seiffert and Granstein, 2002). Furthermore, Saint-Mezard *et al.* (2003) observed in mice that depletion of norepinephrine in peripheral nerve terminals by chemical sympathectomy abolished stress-induced DC migration from skin to draining lymph nodes. These observations suggest that CGRP and norepinephrine released by nerve endings in the skin (reviewed in Legat *et al.*, 2002) may modulate LC frequency.

Similarly, the underlying pathomechanisms of stress-induced modulation of CGRP have not been delineated, although it is known that both glucocorticoids and catecholamines regulate expression of this peptide (Kawasaki *et al.*, 1990; reviewed in Sternberg, 2006). Glucocorticoids upregulate the expression and release of CGRP in dorsal root ganglion sensory neurons *in vitro,* and studies in mice have shown that norepinephrine released from adrenergic nerves inhibits the production and release of CGRP through α_2-adrenoreceptors in sensory neurons of the dorsal root ganglion (Kawasaki *et al.*, 1990). This supports the concept that immune modulation by peripheral neuropeptides occurs via cross-talk between the hypothalamic–pituitary–adrenal axis, autonomic and peripheral nervous systems. It is possible that a stress-induced increase in levels of circulating cortisol and perhaps other stress hormones may be responsible for the observed change in epidermal LC frequency. This and other potential direct and indirect mechanisms form the basis of future studies.

Sex steroid hormones have been implicated in the cutaneous immune response and phase of menstrual cycle in female subjects may influence response to acute social stress. Estrogens may modulate cytokine and chemokine secretion by human DC (Bengtsson *et al.*, 2004) and progesterone is considered a natural immunosuppressor (Cutolo *et al.*, 2004). Furthermore, androgens such as testosterone or androsterone are known to be immunosuppressors. However, both non-stressed controls and 24-hour Trier groups were well balanced in terms of male to female ratio.

At 24 hours, we observed a significant enhancement of immunoreactivity from PGP 9.5+ nerve fibers in the group that had been stressed. We speculate that acute exposure to stress activates nerves, via the neuronal cell body located in the dorsal root ganglion, to produce proteins such as PGP 9.5. However, despite being a reliable marker of nerve fibers in human skin, the precise function of PGP 9.5 is not known and the teleologic reason for the observed increase is unclear at present.

Given the relatively short interval of 4 hours, at which a nonsignificant increase was noted in this relatively small group ($n = 5$), or 24 hours after the baseline skin biopsies, it is unlikely that increased PGP 9.5 expression could be ascribed to nerve growth.

It is recognized that an improved understanding of cutaneous neuroimmunology and the ''brain–skin'' axis in particular may be beneficial to our management strategies for inflammatory skin disease. Consequently, we believe the above-mentioned studies contribute to our understanding of the complex processes that underlie exacerbation of inflammatory skin diseases by stressful life events.

MATERIALS AND METHODS

Clinical protocol

The study protocol was approved by an independent local research ethics committee (Central Manchester, Manchester, UK). This study was conducted according to the Declaration of Helsinki Principles. All volunteers gave written, informed consent before participation in the study.

Volunteers were recruited and interviewed over an 11-month period from August 2005 until July 2006. All volunteers had to comply with inclusion and exclusion criteria that, briefly, included: (i) age between 18 and 55 years; (ii) no history of skin disease, neuropathic disease, or an endocrine disorder; (iii) not taking regular systemic therapy, apart from the oral contraceptive pill; and (iv) gave no history of severe or enduring mental illness. Informed consent was given before participation in the study.

Following recruitment to the study volunteers were sent the PSWQ, a psychometric questionnaire (Meyer *et al.*, 1990), by mail, to complete on the evening before the first testing session.

On day 1, on arrival in the department, all participants had a clinical assessment that included a full medical history, skin examination and baseline blood pressure and pulse measurements. Thereafter, a pair of 6 mm punch biopsies (aseptic technique; local anesthetic, 1% lidocaine) was taken from sun-protected buttock skin. A second pair of biopsies was taken from the contralateral buttock either 4 or 24 hours after exposure to the acute social stressor – modified Trier paradigm – see below. Controls were not exposed to the Trier stressor and had biopsies taken at baseline and 24 hours later – this also addressed the issue of biopsy-induced stress.

At the end of the first study visit, following the stressor (24 hours Trier) or baseline biopsies (controls), blood pressure and pulse rate measurements were taken and participants used a linear VAS to record the amount of stress experienced by participation in the study. This scale ranged from ''0'' (''not at all'') to 100 (''very much''). One pair of skin biopsies (baseline and 4 or 24 hours) was processed for epidermal sheets and quantification of epidermal LC densities using immunofluorescence staining for CD1a (see below). The second pair of biopsies (baseline and 4 or 24 hours) was used for immunohistochemistry to examine expression of PGP 9.5 and CGRP as well as the association between CGRP+ fibers and LC.

Modified Trier paradigm

We used the modified Trier paradigm to induce social stress in healthy, human volunteers. The Trier Social Stress Test, first described by Kirschbaum *et al.* (1993), is a well-validated psychosocial research tool. An adjusted version, the modified Trier paradigm, involves delivering a short speech to an audience of ''experts'' and it too has been shown to be a valid, reliable tool to evoke social stress (Buske-Kirschbaum *et al.*, 2002, 2007; Richards *et al.*, 2005). Moreover, significant increases in epinephrine, norepinephrine, and cortisol occur in response to the Trier stress test (Rohleder *et al.*, 2006).

Participants were provided with standard information about car theft and then given 5 minutes to prepare a short, 5-minute speech on the topic of ''Car thefts in the UK''. They were informed that they would present to an audience of two people whom they believed were experts in the techniques of oral presentation.

PSWQ

Pathological worrying was assessed by the PSWQ (Meyer *et al.*, 1990). This is a 16-item scale that has been shown to be a valid and reliable measure of pathological worry (Meyer *et al.*, 1990). Items are rated on a 1–5 scale (1, ''not at all typical of me'', to 5, ''very typical of me''). Our previous studies have shown that these measures discriminate reliably between individuals with high and low psychological stress and distress (Fortune *et al.*, 2003).

Preparation and analysis of epidermal sheets

One pair of biopsies was processed for analysis of epidermal $CD1a^+$ LC. Biopsies were placed immediately in 0.02 mol l^{-1} ethylenediamine tetraacetic acid (Sigma, St Louis, MO) dissolved in phosphate-buffered saline (PBS) and incubated for 2 hours at 37°C. Forceps were used to separate the epidermis from the dermis. This was washed in PBS and fixed in acetone for 20 minutes at −20°C. After being washed in PBS, epidermal sheets were incubated at room temperature for 30 minutes with a monoclonal antibody specific to CD1a (clone NA1/34 (mouse IgG2a); Dako Ltd, Cambridge, UK) diluted to 10 μg ml^{-1} in PBS containing 0.1% bovine serum albumin (BSA). Sheets were washed in PBS prior to incubation for 30 minutes with FITC-conjugated goat $F(ab')_2$ anti-mouse immunoglobulins (Dako) diluted 1:100 in 0.1% BSA/PBS. Finally sheets were washed in PBS and mounted on microscope slides in Citifluor (Citifluor Ltd, London, UK) and sealed with nail varnish (Griffiths *et al.*, 2001).

Biopsy preparation for analysis of PGP 9.5 and CGRP

The other pair of biopsies was stored in cold Zamboni's solution (4°C for 24 hours) before being transferred to 15% sucrose in PBS, which was changed daily until the tissue was saturated (usually 2 days).

Immunohistochemistry. PGP 9.5 and CGRP were visualized in 14-μm sections (OFT cryostat, Bright instruments, Cambridge, UK). For visualization, triplicate sections were solubilized in 0.2% Triton detergent for 60 minutes. After being washed in PBS, the sections were placed in a PBS solution containing 5% Pontamine Sky Blue (BDH, Poole, Dorset, UK) and 10% DMSO to decrease background autofluorescence. Following further washes, sections were blocked with 1% normal goat serum for 45 minutes at room temperature before application of well-characterized primary antibodies in diluent solution (PBS with 0.1% sodium azide, 1% goat serum, and 0.03% Triton). The primary antibodies were polyclonal rabbit anti-human PGP 9.5 (Affiniti Research Products Ltd, Exeter, UK; dilution 1:1,500) or polyclonal rabbit anti-human CGRP (Affiniti; 1:1,000). Incubation was performed overnight at 4°C. After rigorous washes in PBS to remove unbound antibody, sections were incubated with a fluorescein-conjugated polyclonal goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA; 1:100) for 1 hour at room temperature. Sections were mounted with Vectashield™ (Vector Laboratories).

Double-labeled immunofluorescence was performed to evaluate possible contacts between immunoreactive nerve fibers and LCs. A monoclonal mouse anti-CD1a primary antibody (DakoCytomation Inc., Carpinteria, CA; 1:100) was used to detect LCs.

Quantitative analysis. The slides were stored in the dark at 4°C to avoid fluorescence fading and analyzed within 48 hours of staining. A random sample of six visual fields (Olympus BX60 microscope, Olympus UK Ltd, London, UK), including both epidermis and papillary dermis (PGP 9.5 and CGRP) was captured at ×20 magnification (Diagnostic instruments, Sterling Heights, MI). Images were analyzed using an automated method of quantifying the area of positive staining in each field of view (Image Pro-Plus, Media Cybernetics, Silverspring, MD). The area of interest (epidermis and papillary dermis) was outlined by tracing its margin. Artifacts and strong autofluorescence were digitally edited before analysis. Nerve fiber density was expressed as percentage immunostained area per field.

Confocal microscopy

Confocal laser scanning microscopy was used to confirm the contacts between nerve fibers and LC. Immunofluorescence staining was documented as digitized false-color images obtained with a Bio-Rad MRC1024 Multiphoton System coupled with an inverted Nikon microscope (Carl Zeiss MicroImaging Inc., Oberkochen, Germany formerly Bio-Rad Cell Science, CA). Confocal assistant version 4.02 (CAS) was used to compose and label plates from single images without manipulation of contrast or brightness.

Statistical analysis

LCs. Stress levels were considered by analysis of variance allowing for subject as a random effect, separately for the 24 hours control, 24 hours stressed, and 4 hours stressed groups. Twenty-four hours stress levels were considered by analysis of variance and analysis of covariance on the naive stress level.

Statistical analysis was conducted using the MIXED procedure in SAS (version 9.1 Buckinghamshire, UK). Least-square means for each group were calculated using the LSMEAN option. Differences between relevant groups were tested statistically by comparing least-square means using a two-sided Student's *t*-test, based on the error mean square in the analysis.

PGP 9.5 and CGRP. The statistical significance of differences in PGP 9.5 and CGRP expression between the control non-stressed and Trier stress groups was evaluated using the independent *t*-test (equal variances not assumed) (SPSS software; SPSS Inc., Chicago, IL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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